REMARKS

Claims 19-22, 25, and 28 have been amended, and new claims 33-110 added, to more particularly point out and distinctly claim that which Applicant regards as the invention. Support for the amendments to claims 19-22, 25 and 28 is found at page 8, lines 14-21 of the specification as filed. Support for new claims 33-56 and 77-100 is found at page 8, lines 14-18, at page 12, lines 20-29, and at page 23, line 1 to page 24, line 4, of the specification as filed. In connection therewith, the Examiner's attention is invited to Exhibit A which documents at page 2 thereof, that the phrase "Con A Sepharose," as used at page 23, line 20 of the specification as filed, is inherently a separation and purification medium comprising Concanavalin A attached to agarose beads, and Exhibit B, which indicates that the term "Sepharose" is a trademark used to describe any products of Amersham Biosciences based on Amersham Biosciences' bead-formed agarose based gel.

Support for new claims 63-74 is found at page 12, lines 20-29; at page 5, lines 19-31; at page 8, lines 14-21 and 31-34; at page 9, lines 9-11; and at page 10, lines 29-33, of the specification as filed. Support for new claim 75 and 76 is found at page 11, lines 1-4 of the specification as filed. Support for new claims 57-62 and 101-106 is found at page 26, lines 16-23 of the specification as filed. Support for new claims 107-110 is found at page 14, line 29, to page 15, line 4, of the specification as filed.

CONCLUSION

Applicant respectfully requests that the amendments and remarks be entered and made of record in the instant application. An early allowance is earnestly requested.

Respectfully submitted,

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Enclosure

EXHIBIT A

instructions

Con A Sepharose 4B

Con A Sepharose™ is Concanavalin A coupled to Sepharose 4B by the cyanogen bromide method.

Concanavalin A (Con A) is a tetrameric metalloprotein isolated from *Canavalia ensiformis* (jack bean). Con A binds molcules containing α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. The binding sugar requires the presence of C-3, C-4 and C-5 hydroxyl groups for reaction with Con A. Con A coupled to Sepharose is routinely used for separation and purification of glycoproteins, polysaccharides and glycolipids. Other application areas where Con A Sepharose 4B has been used are purification of enzyme-antibody conjugates, purification of IgM, isolation of cell surface glycoproteins from detergent-solubilized membranes, separation of membrane vesicles, and the study of changes in composition of carbohydrate-containing substances.

To maintain the binding characteristics of Con A Sepharose 4B, the presence of both Mn^{2+} and Ca^{2+} is essential. These are present in large

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excess in the medium supplied but the protein-metal ion complex remains active and is stable at neutral pH even in the absence of free metal ions. However, to preserve the binding activity of the Con A molecule below pH 5, excess Mn^{2+} and Ca^{2+} (1 mM) must be present. This will ensure an active Con A-metal complex.

Table 1. Medium characteristics

Ligand density:	10-16 mg Con A/ml drained medium
Available capacity*:	20-45 mg porcine thyroglobulin/ml medium
Bead structure:	4% agarose
Bead size range:	45-165 µm
Mean bead size:	90 µm
Max linear flow rate**:	75 cm/h at 25°C, HR 16/10 column, 5 cm bed height
pH stability***.	
Long term:	4-9
Short term:	4-9
Chemical stability:	Stable to all commonly used aqueous buffers. Chelating agents such as EDTA, 8 M urea or solutions having a pH below 3 should be avoided as these conditions results in removal of manganese from the lectin with loss of activity as a result.
Physical stability:	Negligible volume variation due to changes in pH or ionic strength.

* The capacity data was determined in 0.1 M phosphate buffer pH 7.0.

** Linear flow rate = $\frac{\text{volumetric flow rate (cm}^3\text{/h)}}{\text{column cross-sectional area (cm}^2\text{)}}$

*** The ranges given are estimates based on our knowledge and experience. Please note the following:
pH stability, long term refers to the pH interval where the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance.
pH stability, short term refers to the pH interval for regeneration.

Preparing the medium

Con A Sepharose 4B is supplied pre-swollen in 0.1 M acetate buffer pH 6 containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$. 20% ethanol is added as a preservative. Wash the required amount of medium with 10 bed volumes of binding buffer to remove the preservative. Prepare a slurry with binding buffer in a ratio of 75% settled medium to 25% buffer. The binding buffer should not contain agents which significantly increase the viscosity. The column may be equilibrated with viscous buffers at reduced flow rates after packing is completed.

Packing Sepharose 4B

1. Equilibrate all material to the temperature at which the chromatography will be performed.
2. De-gas the medium slurry.
3. Eliminate air from the column dead spaces by flushing the end pieces with buffer. Make sure no air has been trapped under the column net. Close the column outlet with a few centimeters of buffer remaining in the column.
4. Pour the slurry into the column in one continuous motion. Pouring the slurry down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
5. Immediately fill the remainder of the column with buffer, mount the column top piece onto the column and connect the column to a pump.

6. Open the bottom outlet of the column and set the pump to run at the desired flow rate. This should be at least 133% of the flow rate to be used during subsequent chromatographic procedures. However, the maximum flow rate, see Table 1, is typically employed during packing.

   Note: If you have packed at the maximum linear flow rate, do not exceed 75% of this in subsequent chromatographic procedures.

7. Maintain the packing flow rate for 3 bed volumes after a constant bed height is reached.

Using an adaptor

Adaptors should be fitted as follows:

1. After the medium has been packed as described above, close the column outlet and remove the top piece from the column. Carefully fill the rest of the column with buffer to form an upward meniscus at the top.
2. Insert the adaptor at an angle into the column ensuring that no air is trapped under the net.
3. Make all tubing connections at this stage. There must be a bubble-free liquid connection between the column and the pump.
4. Slide the plunger slowly down the column so that the air above the net and in the capillary tubings is displaced by eluent. Valves on the inlet side of the column should be turned in all directions during this procedure to ensure that air is removed.

5. Lock the adaptor in position on the medium surface, open the column outlet and start the eluent flow. Pass eluent through the column at the packing flow rate until the bed is stable. Re-position the adaptor on the medium surface as necessary.

The column is now packed and equilibrated and ready for use.

Binding

Binding of glycoproteins and carbobydrate containing proteins occurs at neutral pH. The binding of substances to Con A Sepharose 4B requires the presence of both Mn^{2+} and Ca^{2+}. The protein-metal ion complex remains active and is stable at neutral pH even in the absence of the free metal ions. However to preserve the binding activity of the Con A molecule below pH 5, excess Mn^{2+} and Ca^{2+} (1 mM) must be present. This will ensure an active Con A-metal complex.

Recommended binding buffer is 20 mM Tris-HCl pH 7.4 containing 0.5 M NaCl to avoid non-specific ionic interactions.

Elution

Elution of bound substances can be achieved using an increasing gradient (linear or step) of α-D-methylmannoside or α-D-methylglucoside. These sugars act as strong eluents. Many substances elute at 0.1-0.2 M but higher concentrations may be required for more tightly bound substances. Glucose and mannose may also be used but are weaker eluents.

Tightly bound substances may also be eluted by lowering the pH, but not below pH 4.

Borate is known to form complexes with cis-diols on sugar residues and thus act as an competing eluent. For elution with borate, use a 0.1 M borate buffer, pH 6.5.

Regeneration

Con A Sepharose 4B may be regenerated for re-use by washing the medium alternatively with 2-3 column volumes of high pH (8.5) and low pH (4.5) buffer solutions containing 0.5 M NaCl. This cycle should be repeated 3 times followed by re-equilibration in binding buffer.

All strongly bound substances may not elute during the regeneration procedure. In difficult cases use a detergent (0.1% non-ionic) in a 0.1 M borate buffer, pH 6.5, at low flow rate. A 20% ethanol wash or a gradient wash with up to 50% ethylene glycol may be used to elute even the most strongly bound substances.

An alternative method for regeneration the medium is to wash with a detergent solution, e.g. 0.1% Triton™ X-100 at 37°C for one minute.

In both cases, re-equilibrate with at least 5 bed volumes of binding buffer.

Storage

Store the medium at 4-8°C in 0.1 M acetate buffer pH 6 containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$. Use 20% ethanol as preservative.
Con A Sepharose 4B must not be frozen.

Further information

Check www.chromatography.amershambiosciences.com for more information. Useful information is also available in the Affinity Chromatography Handbook, see ordering information.

Ordering information

Product	Pack size	Code No.
Con A Sepharose 4B	5 ml	17-0440-03
Con A Sepharose 4B	100 ml	17-0440-01
Literature		
Affinity Chromatography Handbook, Principles and Methods	1	18-1022-29

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EXHIBIT B

What is the difference between Sepharose™ and Agarose?

Sepharose is an Amersham Biosciences trademark name for any of our products based on our bead formed agarose based gel. Agarose is a generic term referring to part of the complex mixture of charged and neutral polysaccharides known as agar. The agarose used to make Sepharose is obtained by a purification process that removes charged polysaccharides to give a gel with only a very small number of residual charged groups. When comparing a Sepharose product to an agarose product, you should also consider the following:

Type of ligand, its concentration on the gel, and its orientation (how is it attached?). This will affect the capacity of binding for a particular target..

Agarose content (percent) and degree of cross-linking. This will affect the ability of the gel to be used in columns and with a LC system..

You may also find more information on this topic on our **E-Tech Support** section.